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PI: Prof. dr. Willem J. DeGrip

Address: Katholieke Universiteit Nijmegen
Dept Biochemistry – FMW 160
Institute of Cellular Signalling
P.O. Box 9101
6500 HB Nijmegen
The Netherlands

Phone: +31-24-3614263 (off)/3614259 (secr)
Fax: +31-24-3540525
Email: wdegrip@baserv.uci.kun.nl

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FINAL REPORT

Title: "A novel visual pigment family: Circadian implications".

Objectives

- Production of an antiserum against melanopsin, a putative circadian photoreceptor, permitting analysis of its expression pattern in mammalian retina.
- Heterologous expression of melanopsin using recombinant baculovirus for a first characterization of its biochemical properties.

Background

Human performance is under tight control of a daily rhythm (circadian activity), which regulates the quite significant change in physiological activity during day and night time. This rhythm is entrained to the daily light/dark cycle by a photoreceptor system, which in mammals has still not been identified. Disturbance of this rhythm, e.g. by crossing of time zones, leads to severe physiological imbalance, until it has been reset (phase-shifted) to the new conditions by the photoreceptor system. Proper intervention in this process requires identification of the responsible photoreceptor and its signaling pathways.

Quite recently, a novel photopigment (melanopsin) has been discovered by recombinant DNA techniques first in *Xenopus* melanophores and retina [1] and subsequently also in mammalian retina. Melanopsin is a candidate for a new class of cellular signaling molecules involved in regulating circadian activity. Adjustment of environmental lighting to optimally activate this pigment or modulating the activity of this pigment and/or its transduction pathway by specific drugs could permit control of phase shifts without adverse effects on vision or other related signaling pathways. Our objective for this proposal is to lay the groundwork for detailed characterization of properties and species and tissue distribution of melanopsin.

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Dr. Willem J. de Grip

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Katholieke Universiteit Nijmegen
PO Box 9101
Nijmegen NL-6500
Netherlands

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13. ABSTRACT (Maximum 200 words)

This report results from a contract tasking Katholieke Universiteit Nijmegen as follows: The contractor will characterize properties and species and tissue distribution of the novel photopigment melanopsin.

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Results

1. Antiserum production

Preparation of antigen

As pure melanopsin cannot yet be prepared, we decided to use a common alternative to generate an antigen: a highly selective peptide sequence from the protein, coupled to a suitable carrier to increase the antigenic response. A 15-mer sequence FLAIRSTRGRMVQKLG (Flair-peptide for short), that represents residues 216-230 of melanopsin, was selected. This has good antigenic potential [2], is located in a putative loop-region and is highly characteristic for and rather well conserved within melanopsin of several species (no other close hits in the database). As carriers we decided to use KLH (keyhole limpet hemocyanin) as well as RSA (rabbit serum albumin).

About 2.5 mg of KLH-Flair conjugate was kindly provided by Dr. M. Rollag (USUHS, Bethesda, MD, USA). RSA-Flair conjugate was prepared using the SATA (succinimidoyl-acetyl-thioacelyl)-MHS (maleimido hexanoyl-N-hydroxysuccinimide) coupling [3], which is reported to generate a linker group between carrier and peptide of very low antigenicity. The coupling index (number of peptides conjugated to one carrier) was determined by amino acid analysis following total acid hydrolysis [3]. In this way we prepared about 10 mg of RSA-Flair with a coupling index of 5 ± 1.

Immunization

In our experience, one out of three rabbits produces a high-titer antiserum when repetitively challenged with 0.1 - 0.2 mg of protein antigen. Therefore a set of three rabbits (registration nr: 9-71, 9-72 an 9-73) were used for immunization with KLH-Flair and a set of three (9-74, 9-75 and 9-76) with RSA-Flair. In our Central Animal Facility female New Zealand rabbits at an age of about 3 months and a weight of 2 kg are routinely used for this protocol. Animals were primed with 0.2 ml of an 1:1 mixture of Freund Complete Adjuvant (FCA) and antigen solution (0.15 mg carrier-Flair conjugate), by subcutaneous injection of 0.05 ml at 4 sites on the back. Booster injections, similar to priming but without FCA, are given 4 weeks after priming and subsequently at 2 week intervals. At the second and subsequent boosters a 2 ml serum sample is drawn to follow the development of the antiserum titer. All manipulations follow the Dutch national code of practice.

We have proceeded up to the fifth booster. Usually it takes at least four boosters before titers level. Titers did not increase significantly after the fifth booster, and the animals were bled three weeks after this booster had been given.

Titer-development

Titers were determined by enzyme-linked immunosorbent assay (ELISA) [4], using RSA-Flair as immobilized antigen. Titers of RSA-Flair rabbits were corrected for reaction with RSA or linker by a second ELISA using an unrelated RSA-peptide conjugate as immobilized antigen. The titer is defined as the dilution that gives 50% of the maximal binding, measured as OD_{492} units [4].

All blood samples of all rabbits have meanwhile been titered. The results are shown in Table 1. Two of the three rabbits challenged with the KLH-Flair conjugate (9-71 and 9-72) generated good titers (6000 and 5000, respectively). The RSA-conjugates generated very little immune response. Only rabbit 9-76 produced antiserum with a modest titer (1000).

Immunocytochemistry

Although higher titers are preferable to minimize background due to aspecific interactions, early trials were performed with the 2nd blood sample of of rabbit 9-71 (9-71-2). Sections of mouse retina, kindly provided by Dr. J. Janssen, Ophthalmology, were incubated with dilutions of 9-71-2 varying between 1:50 and 1:250 and processed for fluorescence microscopy

[5]. All dilutions gave a strong background reaction. We then postponed further studies until sera of higher titer were obtained.

Further tests were performed with the highest-titer sera 9-71-4, 9-71 (final) and 9-72 (final). A specific reaction could still not be obtained. Low dilutions (1:≤250) resulted in a high background, and at higher dilutions (1:≥500) no specific signal above the low background was observed.

Western blotting

All antiserum samples with a titer ≥1000 were tested on blots of ocular extracts from rat and *Xenopus*, after 1D-analysis by means of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) performed as described [1]. In all cases a fairly similar pattern was observed (Figure 1) consisting of a number of bands in the 35-65 kDa range. Remarkably, the strongest reaction was observed after the second booster (sera 9-71-2 and 9-72-2) and it decreased in subsequent sera samples, in spite of a higher titer for the conjugated peptide. A specific band in the 55-60 kDa region, potentially corresponding to melanopsin, could not unambiguously be identified.

Purification of antisera

Although the antisera are able to detect overexpressed recombinant melanopsin in Western blots (see below), they display too strong aspecific interaction with tissue components to allow detection of the very small amounts of native melanopsin in tissue extracts or sections. We attempted to improve the specificity by chromatography over RSA-Flair immobilized onto CNBr-Sepharose (Sigma), according to the manufacturer's instructions. Trials with 9-71-4 indicated, that over 70% of the RSA-Flair binding activity, as measured by ELISA, did bind to the column. However, this antibody population could only be partially and under very harsh conditions (0.1% Ammonyx LO, 0.5 M betaine, pH. 2.5-3.0) eluted again. The eluted fractions were rapidly stabilized (neutral pH, 0.5% BSA), but actually performed worse than the mother serum (much weaker reaction with recombinant melanopsin, still appreciable background). Possibly, the major epitope in the Flair-conjugate is not present or not very well accessible in the intact protein.

Further attempts to purify the antisera were abandoned. Alternative approaches, that might generate better quality sera would be to immunize either with the complete purified protein, or with a fusion-protein containing a larger fragment, e.g. the C-terminal part fused to glutathione-S-transferase or maltose-binding-protein, or with an expression plasmid containing the entire coding-sequence of melanopsin (genetic immunization). We have started preparations to follow up along these lines, but those experiments fall outside the scope of this contract.

2. Expression of melanopsin with recombinant baculovirus

Generation of recombinant baculovirus

Full-length c-DNA of *Xenopus* melanopsin was kindly provided by Drs. I. Provencio and M. Rollag (USUHS, Bethesda). We transferred it by standard cloning procedures into the baculovirus transfer vector pVL1393. Cotransfection with baculogold DNA (Pharmingen, San Diego, CA, USA) into the insect cell line Sf9 (ATCC CRL-1711) generated recombinant baculovirus with the melanopsin coding sequence under control of the very strong late-phase polyhedrin promoter. Techniques were as described before [6]. Correct recombination was checked by PCR and recombinant virus was plaque-purified and expanded in Sf9 cells to a high-titer stock (10^7-10^8 pfu/ml).

Expression of recombinant protein

Procedures followed those described earlier [6]. Sf9 cells grown in small culture flasks were infected in mid-logarithmic phase with the melanopsin-baculovirus at a multiplicity of infection (MOI) of 0.1. Cells were harvested four to five days after infection, lysed by hypotonic treatment and the membrane pellet subjected to SDS-PAGE for immunoblot analysis. All available

antisera samples were tested. Only those with a titer over 1000 produced a reasonably clear, distinct pattern, which was quite similar for all samples (Figure 2, lane 1). The most distinct band (arrow) travels with an apparent Mw of 55 ± 3 kDa. This band also shows a weak reaction with the anti-rhodopsin antiserum CERN886, which explains its detection in *Xenopus* eye extracts [1]. A Mw of 55 ± 3 kDa agrees with the size reported for *Xenopus* melanophores [1] and is in the correct range for full-length *Xenopus* melanopsin (60.3 kDa). Membrane proteins usually migrate with a smaller apparent Mw in SDS-PAGE (5-10 kDa less) due to enhanced SDS-binding. No reaction is detected in cells infected with a rhodopsin-baculovirus (Figure 1, lane 2). The data demonstrate, that the antisera not only react with the peptide conjugates, but also do recognize the protein. In addition, they convincingly show, that full-length protein can be expressed using the baculovirus system. These results could be reproduced in larger-scale experiments (100-250 ml spinner bottles [6,7]).

Recombinant photopigments are produced as the apoprotein and require binding of their ligand (regeneration with the chromophore) to regain photosensitivity. The natural ligand of vertebrate visual pigments is 11-*cis* retinal, but they also often react with 9-*cis* retinal generating "iso-pigments" [8], and in the opsin family all-*trans* retinal also can serve as a ligand [9].

The low sequence homology of melanopsin with other opsins [1] forbids to predict which ligand it might use or where in the visible range its absorbance band would be located. Therefore we incubated the Sf9 cell membrane fraction containing recombinant melanopsin in the dark with either retinal isomer (11-*cis,* 9-*cis,* and all-*trans*) and checked for the presence of a photosensitive protein by UV/Vis difference-spectroscopy, hydroxylamine treatment and acid denaturation [6-10]. A variety of incubation conditions was tested (1, 10, 50 μM retinal isomer; 0, 0.05, 0.2% (w/v) dodecylmaltoside; 0, 0.2, 0.4% (w/v) CHAPS; 10 mM β-cyclodextrin; 20% (v/v) glycerol; 10, 150, 500 mM NaCl; 0.5, 1.0 M betaine; pH. 5.5, 6.5, 7.5.), but we could never detect a trace of a photosensitive pigment.

The most likely explanation is, that the interaction of melanopsin with its ligand more closely resembles that of invertebrate pigments, to which it shows higher sequence homology than to vertebrate opsins [1]. While the latter spontaneously regenerate with their ligand [8], the former only very reluctantly do so, and as recombinant proteins so far have always been refractory to reveal their photosensitive holo-nature. Probably specific ligand-binding factors are require to chaperone this reaction, but these have not yet been identified.

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SIGNATURE

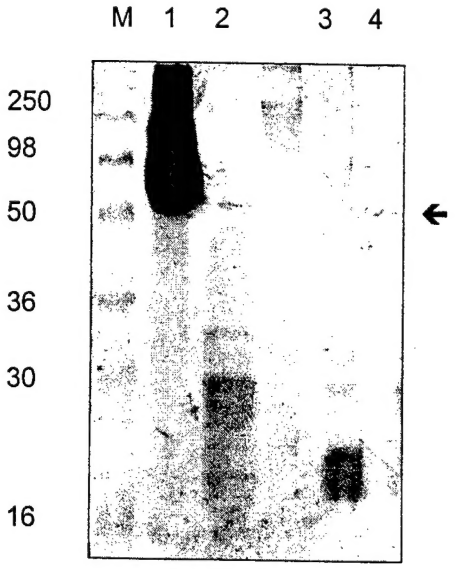
Prof. dr. Willem J. DeGrip

Table 1. Titer development in rabbits challenged with melanopsin Flair-peptide conjugates[a]

rabbit reg. nr.[b] / blood sample [c]	9-71	9-72	9-73	9-74	9-75	9-76
1	200	100	100	< 50	< 50	< 50
2	2000	1000	400	< 50	< 50	100
3	2500	3000	400	< 50	< 50	400
4	5000	4500	1500	< 50	< 50	800
final	6000	5000	2000	< 50	200	1000

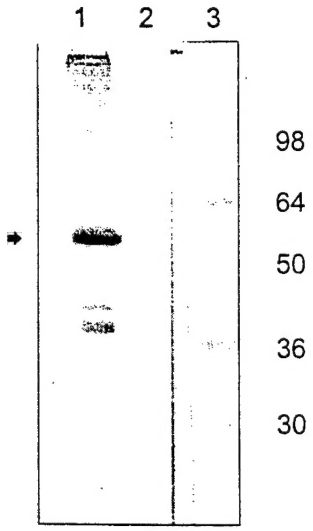
[a] Titers were determined by ELISA [4] against immobilized RSA-Flair.
[b] Rabbits 9-71, 9-72 and 9-73 were challenged with KLH-Flair conjugate, rabbits 9-74, 9-75 and 9-76 with RSA-Flair conjugate.
[c] First blood samples were drawn during second booster. Thereafter, samples are drawn at the subsequent biweekly boosters. Pre-immune sera only show background binding to RSA-Flair.

Figure 1. Western blots of ocular extracts



Ocular SDS extracts of rat (*lane 2*), *Xenopus* (*lane 3*), or chicken (*lane 4*) were analyzed by SDS-PAGE and probed for melanopsin by immunoblotting with antiserum 9-73 (final). Several bands are observed in the 30-98 kDa region. A band in the correct size range for melanopsin is detectable in the rat and chicken extracts (arrow). A strong reaction is always found for the RSA-Flair conjugate (*lane 1*). In rat and chicken extracts often a relatively strong reaction of unknown origin is observed in the 16-30 kDa region. The same pattern was observed with all other sera in Table 1 with titers ≥1000, be it that the reaction with earlier samples (9-71/2, 9-71/3 and 9-72/3) was more pronounced. *Lane M* presents proteins for size calibration (Mw in kDa indicated).

Figure 2. Melanopsin expression



Expression of recombinant *Xenopus* melanopsin using baculovirus. Membrane extracts from Sf9 cells infected with melanopsin-baculovirus (*Lane 1*) or rhodopsin-baculovirus [7] (*Lane 2*) were probed with antiserum sample 9-71-2. The same pattern was observed with all other sera with titers >1000. *Lane 3* presents proteins for calibration (Mw in kDa indicated).

The main band in *lane 1* (arrow) corresponds to a protein of 55 ± 3 kDa, which agrees with the size reported for *Xenopus* melanophores [1] and is in the correct range for full-length melanopsin (60 kDa).